OPTIMIZATION of YELLOW PIGMENT PRODUCTION by *Monascus purpureus* from BANANA PEEL

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ABSTRACT

The purpose of this research is mainly to study the optimization of yellow pigment production by *Monascus purpureus* from banana peel. There has been an increasing trend towards replacement of synthetic colorants with natural pigments in last decades due to the high demand of natural products. Bacterial pigments are considered as an alternative to produce natural colour. Production of yellow pigment of *Monascus purpureus* grow on agricultural waste that is banana peels was studies. From the study, the optimum growth temperature of *Monascus purpureus* and pigment production is at 30°C, 60 % moisture content and optimum pH is 5.5. The pigment extracted was with ethanol as a solvent. Measurement of yellow pigment carried out using UV-VIS 1800 spectrophotometer at 400 nm. The relation of three parameters, were further studies on Response Surface Methodology. By the point prediction tool of Design-Expert 6.7, the optimum values of the factors for maximum pigment production were determined: initial substrate moisture content 57.5%, pH 5.5 and temperature 40˚C. With this point optimization, the pigment yield for yellow pigment is 2.2104 OD/gram dry solid was closely to 2.3754 OD/gram dry solid with is the predicted yield.

OPTIMUM PENGELUARAN PIGMEN KUNING DARIPADA Monascus purpureus OLEH KULIT PISANG

ABSTRAK

Tujuan kajian dilakukan adalah untuk mengkaji optimum pengeluaran pigmen kuning daripada *Monascus purpureus* oleh kulit pisang. Terdapat peningkatan hala tuju dalam beberapa dekad ini terhadap penggantian pewarna sintetik dengan pigmen asli disebabkan peningkatan permintaan pengguna terhadap produk-produk asli dan pigmen daripada bakteria dianggap sebagai alternatif kepada pewarna sintetik. Penghasilan dan pengekstrakan pigmen kuning oleh *Monascus purpureus* yang dikulturkan di atas sisa pertanian iaitu kulit pisang telah di kaji. Daripada kajian ini, suhu optimum untuk pertumbuhan dan penghasilan pigmen oleh *Monascus purpureus* ialah pada 30°C, kandungan kelembapan 60% dan pH optimum ialah 5.5. Pigmen tersebut diekstrak menggunakan etanol sebagai pelarut. Pencirian pigmen kuning ini dilakukan dengan menggunakan spektrofotometer UV-VIS 1800 pada 400 nm.Response Surface Methodhology telah dipilih untuk kajian selanjutnya. Dengan alat ramalan titik Pakar Rekabentuk-6.7 (Design- Expert 6.7), nilai factor optimum untuk pengeluaran pigmen maksimum telah ditentukan: kandungan lembapan 57.5%, pH 5.5 dan suhu 40 ˚ C. Dengan pengoptimuman ini, hasil pigmen pigmen kuning 2.2104 OD / gram pepejal kering (Od/gds) adalah dekat dengan 2.3754 OD / gram pepejal kering (OD/gds) hasil yang diramalkan.

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CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

For several decades, both natural dyes and synthetic dyes were widely used in various fields of everyday life such as food production (Pandey *at el*., 2001), textile industries, paper production, agricultural practices and studies, water science and technology (Tibor, 2007).

In 2007, the value of the international food colorant market was estimated at around $1.15 billion USD, up 2.5% from $1.07 billion USD in 2004 (Mapari *at el*., 2010). The use of food dyes as supplement material in food industries is extremely beneficial for both the manufacturers and users in deciding the admissibility of processed food (Spears *at el*., 1988; Griffiths, 2005). Possible reasons for consumption coloring in food substances is to preserve the original meals appearance even after processing and storage, to make sure color uniformity to prevent a seasonal variation in tone color, to intensify the regular color of the food and to maintain quality, enhancing color ordinary food and therefore to maintain quality, taste and glow to safeguard vitamin even when exposed to the and boost acceptance food as tempting item (FNB Food Colors, 1971).

Predominantly natural coloring are environmentally friendly, biodegradable, much less toxic and fewer allergenic than synthetic coloring agents. However, research has shown that certain of natural dyes might have a mutagenic impact detected like elderberry color, safflower yellow and carmine that can causing asthma through continues inhalation, but it arguably that most of the natural dyes secure and some have healing impact for example curcumin in turmeric has antibacterial properties (Ali *et al*., 2007). Gardenia yellow (Sato *et al*., 2007) and safflower yellow is natural color. Both of them are low in toxicity, but are subject to the season change, resulting in an unstable industrial production. Although natural dyes have several advantages but there are some limitations as well such tedious extraction of colorings component from the raw materials with low colorings component. For example, in order to obtain 14 g of the dye about 1200 molluskes are needed. Besides that, it also limited number of suitable dyes because it allows only dyeing wool, natural silk, linen and cotton (Sato *et al*., 2007).

According to green technology curriculum, less toxic products and more natural starting material is favorable for today's production lines .In case of dyes, it is well known that synthetic dyes that derived from minerals likes lead chromate and copper sulphate may cause serious health problem (FDA/IFIC, 1993) and environment hazardous effect (Francis, 1987) such as can prevent sunlight penetration decreasing photosynthetic activity in aquatic environment (Yogendra, 2008). Therefore, within the past several decades, synthetic addictives was criticized and consumer refusal shown towards this product (Francis, 1987). During the late 1960s in the United States, environmentalists makes several demonstrating against the use of synthetic coloring agents and these behavior has dispersed widely (Carvalho *et al*., 2003). Therefore, an extensive research was carried out to look for an alternative in order to make sure dyes output and consumption will meet the requirements of the environment and the highly secured (Georgeta *et al*., 2004).

To overcome this limitation, other biological sources such as fungi which includes moulds and yeast, bacteria, algae and plant cell cultures have been used (Saintis *at el*., 2005). There are a number of microorganisms declared have the ability to producing pigment in a high yield, includes Monascus species, Paecilomyces, Serratia, Cordyceps, Streptomyces and Penicillium (Hajjaj *et al.*, 2000). Among them, various species *Monascus* has drawn special attention as they have the capabilities producing vary colored pigment and indicate high chemical stability (Mak *et al*., 1990;. Yongsmith *et al*., 1994; Hamdi *et al*., 1997; Hajjaj *et al*., 2000). *Monascus* pigments were reported for producing non-toxic and may enhance the appearance of food coloring (Vidyalakshmi *et al*., 2009).

In order to produce pigments, *Monascus purpureus* have to be cultured under specific conditions. Two types of culture techniques are used which are solid state fermentation (SSF) and submerged fermentation (SmF). *Monascus* pigment production by submerged and solid-state cultures in complex media has been thoroughly studied (Mak *et al*., 1990; Johns and Stuart, 1991; Yongsmith *et al*., 1994). Biopigments produced by fermentation present a great potential for food applications (Carvalho *et al*., 2005) and have the capacity to increase the marketability of products (Tibor, 2007) because they are natural, produced quickly when compared with vegetable or animal pigments and can be produce at any time of the year (Carvalho *et al*., 2005). They also display advantageous biological activities as antioxidants and anticancer agents.

1.2 IDENTIFICATION OF PROBLEM

The most important issue regarding natural pigment is the price of final product which is more expensive than cheap synthesized dye. According to Diana et al., (2005), the main disadvantages of natural dyes are their extraction yield factors (a few gram of pigment per kg of dried raw material). This makes their current market price about US$1/g, thus limiting their application.

Meanwhile, the use of synthetic dye has several disadvantages which have a potential of carcinogenicity (Fabre *et al*., 1993; Tibor, 2007), ambient pollution possibility and increase of the cutaneous allergies for the user of the product even it is cheaper

Green technology is leading all producers to go towards ecological and less polluted products with fewer by-products; in the case of synthesized dye, natural pigments can be considered as an ideal alternative. Beside that, less toxic products and more natural starting material is favorable for today's production lines (Francis, 1987; Yogendra, 2008).

In this research possibility of using cheap growth media (agricultural wastes) such as banana peel which leads to inexpensive and competitive product, will be been studied with *Monascus purpureus* as original stain.

1.3 STATEMENT OF OBJECTIVES

To studying the effect of different parameters which are initial substrate moisture content, pH and temperature in order to optimize yellow pigment production by *Monascus purpureus* from banana peel.

1.4 SCOPE OF STUDY

In order to achieve the objective, 4 parameter are studies:

i. Effect of different substrate pH on yellow pigment production. The pH range is 4.5, 5.0, 5.5, 6.0 and 6.5.
ii. Effect of different temperature on pigment production. The temperature range is 30°C, 35°C, 40°C, and 45°C, 50 ^{0}C.
iii. Effect of different initial substrate moisture content on pigment production. The range is 45% 50%, 55%, 60% and 65% w/w.
iv. Study the relation of pH, temperature and initial moisture content on yellow pigment of banana peel by optimization method using design expert.

1.5 RATIONAL AND SIGNIFICANCE

This study is believed to provide an optimization method of yellow pigment production by using *Monascus purpureus.* The substrates used in solid state fermentation supply the basic nutrients to the microorganisms and serve as an anchor for the cells. Interestingly, recent studies report that solid state fermentation provides a more adequate habitat for fungi, resulting in high pigment production in a relatively low-cost process when agro-industrial wastes are used as substrate.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The Chinese medical book published in the first century described Anka (also called as anka-kak) or red mold rice as a health benefits. Later, literature documented the use of red color obtained from anka for colouring foods (Khairak *et al*., 2000). In 1884 a French Botanist Philippe van Thieghem isolated the fungus, a red mold growing on potato and and called it as *Monascus ruber*. According to Went (1895), Monascus purpureus from anka obtained from the market of Java and Indonesia. The interest for the color resulted in the characterization of several *Monascus* species isolated from different foods around the world. Presently, there are over 50 patens describing the use of *Monascus* pigments for food. The annual consumption of pigment in Japan approximately 100 tons valued at $1.5 million (Laurent Duffose *et al*., 2005).

Monascus moulds have been commercially employed for the production of major red pigment (Yongsmith *et al*., 1993).However, in recent years; two novel yellow pigments produced *by Monascus sp*. KB20M10.2 (Sato *et al.*, 1992) have been discovered. It was found that *Monascus sp.* KB11304 fermentation produced yellow and red pigments respectively. When strain KB11304 was UV-irradiated to improve its pigment production, the mutant KBM10M16 produces 1.5 times more red pigments than KB11304. When *Monascus* KBM10M16 was itself exposed to UV radiation, Monascus strain KB20M10.2 was obtained which produce yellow pigments (Yongsmith *et al*., 1993). In this paper, yellow pigment production using *Monascus purpureus* strain 5356 was studied.

2.2 DEFINITION OF PIGMENT

The word pigment has a Latin origin and initially denoted a color (in the sense of colored matter), but it was later extended to indicate the colored objects such as makeup. Pigment is defined as the coloring agent in substances which can be produced either by living organisms or chemical reagents. The history of pigment application dates back to prehistoric cave painting, which gives evidence of the use of ocher, hematite, brown iron ore and other mineral-based pigments more than 30,000 years ago (Daniel, 1986).

The most satisfactory way to classify pigment is according to its source, because most of the significant properties which any pigment groups may have in common can be attributed to their composition. They are divided into two major groups which are natural and synthetic pigments. Synthetic pigment is divided into two main groups which are known as organic and inorganic pigments (Daniel, 1986).

2.2.1 Natural Pigment

Naturally pigments are substances generated by living organisms that having color resulting from selected color absorption. A biology pigment includes plant pigments and pigmented animals. A lot of biological structures, like skin, eyes, fur and hair contain pigments such as melanin within specialized cells called chromatophores (Ball, 2002).

2.2.1.1 Importance of Using Natural Pigments

Environmental anxiety regarding synthetic dyes saw resurgence in requests for naturally dyes as naturally dyes are more environmentally friendly than their synthetic counterparts. Natural coloring may showcases better biodegradability and generally having high compatibility with the surroundings (Kamel *et al*., 2005). Lately, the potential for obtaining natural color from the pigmented microbes to be used as naturally dyes are being investigated (Nagia and EL-Mohamedy, 2007). Table 2.1 shows the microbial production of pigments and their status of development (Liu and Nizet, 2009).

It is interesting to note from Table 1.1 that a lot of pigmented production of the bacterial resources is still classified as a research project or in the development stage. Therefore, employed on production of pigment from bacteria should be enhanced particularly in search of affordable and appropriate growth medium which may cut costs and applicable for industrial production.

Table 2.1: Microbial production of pigments (already in use as natural food colorants or with high potential in this field) (Liu and Nizet, 2009).

Microorganism	Pigment	Colour	Status*
		Bacteria	
Agrobacterium aurantiacum	Astaxhantin	Pink-red	RP
Paracoccus carotinifaciens	Astaxhantin	Pink-red	RP
Bradyrhizobium sp.	Canthaxhantin	Dark-red	RP
Streptomyces echinoruber	Rubrolone	Red	DS
Flavobacterium sp.	Zeaxanthin	Yellow	DS
Paracoccus zeaxanthinifaciens	Zeaxanthin	Yellow	RP
		Fungus	
Monascus sp.	Ankaflavin	Yellow	IP
Monascus sp.	Monascorubramin	Red	IP
Penicillium oxalicum	Anthraquinone	Red	IP
Blakeslea trispora	Lycopene	Red	DS
Fusarium sporotrichioides	Lycopene	Red	RP
Cordyceps unilateralis	Naphtoquinone	Deep blood-red	RP
Ashbya gossypi	Riboflavin	Yellow	IP
Monascus sp.	Rubropunctatin	Orange	IP
Blakeslea trispora	ß-carotene	Yellow-orange	IP
Fusarium sporotrichioides	ß-carotene	Yellow-orange	RP

Neurospora crassa	ß-carotene	Yellow-orange	RP
Phycomyces blakesleeanus	ß-carotene	Yellow-orange	RP
Penicillium purpurogenum	Unknown	Red	DS
	Yeast		
Saccharomyces neoformans var. nigricans	Black	Melanin	RP
Xanthophyllomyces dendrorhous	Astaxanthin	Pink-red	DS
Rhodotorula sp.	Torularhodin	Orange-red	DS

*Industrial production (IP), research project (RP)

2.2.2 Synthetic Pigments

A huge number of dyes have been synthesized and used mainly for dying textiles. According to their chemical structure they are generally classified into six classes: Azo, indigoid, anthracene, azobenzene, phtalocyanine, triphenylmethan (trityl).

However, the structural features of dyes sometimes overlapping united in the molecule greater than one element and making it impossible in classification. In addition, besides their use in the textile industry, various dyes have found application in a variety other areas of research up-to-date and industrial activity (Heinrich, 2003).

Due to the German ban on azo dyes, now there are is one step to search a renewable resource to augment requirements for secure dye industry and this trend has led to investigation into the output of natural dyes on a commercial scale (Vankar *et al*., 2007).

2.2.2.1 Effects of Using Synthetic Dyes

a) Effects on Health

In pharmaceutical industry, the synthetic food dye is added in order to add color to many medicinal products, as well as to ensure the same color for all the batches of a given product. Adding a color makes the medicinal product more attractive, easier to recognize, and in some cases, by forming an opaque layer, it stabilizes the ingredients of the medicine which are light sensitive (Jaworska *et al*., 2005).

Although synthetic food dyes are greater long lasting and often cheaper compared the original, however now, using lot of this dye gave rise to the serious bookings regarding health. Some of them, such as tartrazine (E 102),cochineal red (E 124), and sunset yellow (E 110), belonging to the group of azo dyes, can themselves, or in combination with other colorants, provoke allergic or pseudo-allergic reactions (PARs), particularly in people allergic to aspirin and other non-steroidal anti-inflammatory agents, or those suffering from urticaria or asthma (Rowe and Rowe, 1994).

Even supposing the synthetic colorants approved by the Food and Drug Administration (FDA) for use in foods, pharmaceuticals and cosmetic preparations have undergone rigorous scrutiny for their toxicity, surprisingly, a study on the examination of cancer chemo preventive effect of synthetic colorants revealed that, a number of these products were evaluated for their in vitro antitumor promoting effect on Epstein–Barr virus (EBV) antigen induced by tumor promoter 12-*O*-tetradecanoylphorbol-13- actate (TPA). Among these are azo dyes, tartrazine (FD & C Yellow # 5), and derivatives of indigo, indigo carmine (FD & C Blue # 2) (Kapadia *et al*., 1998). For external use synthetic dyes, dye several were withdrawn caused to their clearly hazards. For instance, benzidine dyes may lead bowel cancer, while carbon black, pigment printing ink, regarded as a potential carcinogen.

b) Effects on environment

From the environmental point of view, a great variety of synthetic dyes used for textile dyeing and other industrial applications causes serious pollution when part of these dyes penetrates into waste water during these processes. Most of these compounds are toxic, carcinogenic and highly resistant to degradation (Chung *et al*., 1992).

Azo dye, which is largest group of synthetic dyes, is used extensively in various industries. Although textile mills mostly use them, azo dyes can also be found in food, pharmaceutical, paper and printing, leather, and cosmetics industries. It is not surprising that this compound has become a main environmental anxiety. A lot of these dyes locate their way into environment through the wastewater accessibility. Due to these compounds maintain their color and structural integrity under the disclosures to sunlight, soil, bacteria and sweat, they showcases high obstacles to microbial degradation in wastewater treatment systems (Eichlerová *et al*., 2006).

The synthetic dyes led to the collapse of a huge industry and gave rise to a redistribution of wealth, to the small companies now providing a large proportion

2.2.3 Short History of Yellow Pigments

The oldest yellow pigment is yellow ochre, which was among the first pigments used by humans. Egyptians and the primordial world made wide use of the mineral orpiment for a more brilliant yellow than yellow ochre. In the Middle Ages, Europeans manufactured lead tin yellow. They later imported Indian yellow and rediscovered the method for the production of Naples yellow which was used by the Egyptians. Modern chemistry led to the creation of many other yellows, including chrome yellow, cadmium yellow, lemon yellow and cobalt yellow.

2.2.3.1 Napple Yellow

Naples Yellow**,** or lead antimoniate, has been extremely popular among painters for its pale warmth. Its use as a colorant can be traced back to about 1400

B.C. In its native state, Naples Yellow is a lead-based pigment, and therefore highly toxic.

2.2.3.2 Cobalt Yellow

Cobalt Yellow or Aureolin replaced an earlier pigment called Gamboge, an Asian yellow gum used until the 19th century. Discovered in 1848 by N.W. Fischer in Germany, Aureolin remained popular until the late 19th century, when less expensive, cleaner and more lightfast pigments like the Cadmiums were introduced.

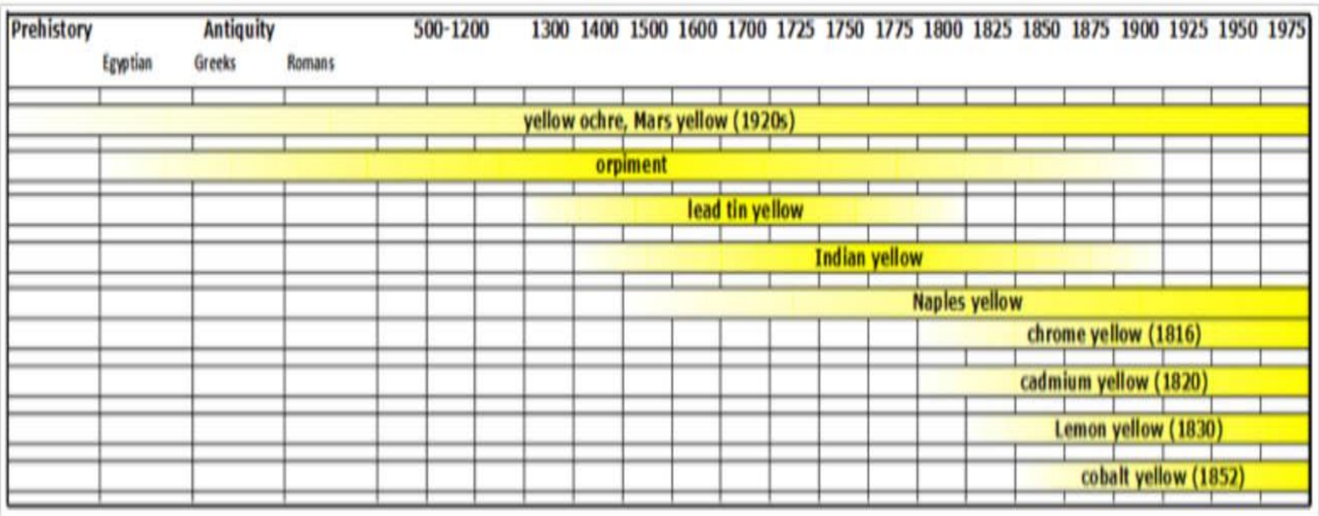

Figure 2.1: Timeline of yellow pigment

2.3 SPECIES AND THE USE OF *Monascus* pigments

The genus *Monascus* can be divided into four species: which are *M.pilosus, M.purpureus, M.ruber and M.froridanus*. *Monascus* belongs to the kingdom of fungi, class of Ascomycetes and family *Monascaceae* in which there are more than thirty kinds now recognized in the world (Juzlova et al., 1999: Chung *et al.* 2006).

Many recent studies show that *Monascus* can produce several fungal metabolic derivatives, such as ethanol, monascus pigments, g-aminobutyric (GABA), monacolins (including monacolin K, dehydromonacolin K, methyl ester of monacolin K hydroxyl-acid from, hydroxyl-acid form of monacolin K, monacolin L, and methyl ester of monacolin L hydroxyl acid form) (Chung *et al.* 2006).

The main uses of *Monascus* in Asian countries especially Japan and China for many centuries are to colour and flavour food such as rice wine, soy bean and beverages. In addition, the interest in pigments produced by *Monascus* sp. in the food industry has been growing because of their wide application (meat, fish, and ketchup) and also due to the carcinogenic and teratogenic effects of some synthetic colorants, like nitrosamines formed from nitrites and nitrates in cured meats (Chen and Johns, 1993: Hamano and Kikilian, 2006). *Monascus* is used in the production of monacolin K which provides the ability to lower blood-lipid levels in animal models and in humans (Endo, 1979).In addition, there is a scientific report on the production of antibiotic activity of *M. purpureus*, on *Bacillus*, *Streptoccus* and *Pseudomonas* (Juzlova *et al*., 1999). *Monascus* sp., a filamentous fungus has been used to make rice wine, soy bean cheese and anka (red rice) in many Asian countries (especially Japan and China) for centuries. *Monascus* spp. is stained easily, and need rigorous culture conditions in fermentation (Chung *et al.* 2006).

2.4 *Monascus purpureus*

Monascus purpureus is a red mold species which may be cultivated on starch containing substrate. The solid state fermentation of rice by Monascus purpureus has a long tradition in East Asian countries (Erdogrul and Azirak, 2004). This mold belongs to the polycetides and has slight bactericidal effects. The production of pigments by this mold was studied and the mixture of pigments are stable from the chemical point if view. As reported, the group includes the orange, yellow and red pigment (Margalith, 1992). *Monascus purpureus* has been well known for red *pigment* production but less study was investigated for yellow pigment production. A monascus purpureus mutant strain-YLCI was obtained for yellow pigment production (Evans and Wang, 1987).

Among the various pigment-producing microorganisms, *Monascus* is reported to produce non-toxic pigments, which can be used as a food colorant. The pigment of *Monascus* improves the coloring appearance of foods (Blanc *et al*., 1995). The optimal cultivation temeperature for this fungal to grow varies from 25 ˚ C to 37 ˚C (Juzlovo *et al*., 1996). Neverthless, the most frequently cited temperature is 30 ˚C.

2.5 PIGMENT FROM *Monascus purpureus*

During growth, *Monascus* spp. breaks down the starch substrate into several metabolites. The structure of pigments as secondary metabolites depends on substrate types and other factor during cultivation such as pH, temperature and moisture